

*Short Communication*

**Generation and Characterization of SULT4A1 Mutant Mouse Models**

Patrick L. Garcia, Mohammed I. Hossain, Shaida A. Andrabi, and Charles N. Falany

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, 35294, USA

**Running title: SULT4A1 mutant mouse models**

**Address correspondence to:** Dr. Charles N. Falany, Dept. of Pharmacology and Toxicology, 1670 University Blvd., Volker Hall Room 151, University of Alabama at Birmingham, Birmingham, AL 35294, USA. Tel (205) 934-9848; FAX (205) 934-8240; E-mail [cfalany@uab.edu](mailto:cfalany@uab.edu)

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**Abbreviations:** AA (amino acid), DIV (Day *in vitro*), HPF (hour post-fertilization), KD (knock-down), KO (knock-out), MO (morpholino), PAPS (3'-Phosphoadenosine-5'-phosphosulfate), PMS (Phelan-McDermid Syndrome), SNP (single nucleotide polymorphism), SULT (Sulfotransferase), SULT4A1 (sulfotransferase 4A1).

## Abstract

Sulfotransferase 4A1 (SULT4A1) belongs to the cytosolic sulfotransferase (SULT) superfamily of enzymes that catalyze sulfonation reactions with a variety of endogenous and exogenous substrates. Of the SULTs, SULT4A1 was shown to have the highest sequence homology between vertebrate species, yet no known function or enzymatic activity have been identified for this orphan SULT. To better understand SULT4A1 function in mammalian brain, two mutant SULT4A1 mouse strains were generated utilizing CRISPR-Cas9 technology. The first strain possessed a 28 base pair (bp) deletion ( $\Delta 28$ ) in exon 1 that resulted in a frameshift mutation with premature termination. The second strain possessed a 12 bp in-frame deletion ( $\Delta 12$ ) immediately preceding an active site histidine<sup>111</sup> common to the SULT family. Homozygous pups of both strains present with severe and progressive neurological symptoms, including tremor, absence seizures, abnormal gait, ataxia, decreased weight gain compared to littermates, and death around post-natal days 21-25. SULT4A1 immunostaining was decreased in brains of heterozygous pups and not detectable in homozygous pups of both SULT4A1 mutants. SULT4A1 localization in subcellular fractions of mouse brain showed SULT4A1 associated with mitochondrial, cytosolic, and microsomal fractions, a novel localization pattern for SULTs. Finally, primary cortical neurons derived from embryonic (E15) CD-1 mice expressed high levels of SULT4A1 throughout the cell except in nuclei. Taken together, SULT4A1 appears to be an essential neuronal protein required for normal brain function, at least in mammals. Mouse models will be valuable in future studies to investigate the regulation and functions of SULT4A1 in the mammalian brain.

## Introduction

In 2000, Falany et al. identified and cloned sulfotransferase 4A1 (SULT4A1) from the brains of humans and rats (Falany et al. 2000). Sequence analysis revealed that SULT4A1 possessed key structural domains characteristic of SULTs, such as the “KXXXFTVXXXE” dimerization domain, an active site histidine (His<sup>111</sup>), and a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) binding site, yet SULT4A1 lacked detectable *in vitro* enzymatic activity (Falany et al., 2000). Immunohistochemical studies showed SULT4A1 to be predominately expressed in neurons of the human cerebral cortex, cerebellum, thalamus, pituitary, medial temporal lobe, choroid plexus, and lentiform nucleus (Liyou et al., 2003). In addition, SULT4A1 is highly conserved among vertebrate species, more so than all other SULTs. Human SULT4A1 shares >97.5% sequence identity with chimpanzee, rabbit, rat, and murine SULT4A1s. Moreover, 118 DNA samples of ethnically diverse people were sequenced for single nucleotide polymorphisms (SNPs) in 10 different SULT genes, and SULT4A1 was the least genetically diverse with only five polymorphisms and none located within the coding region of the gene, suggesting an evolutionary conserved function in vertebrate brain (Allali-Hassani et al., 2007).

Although a validated function or substrate has not been identified, SULT4A1 has been implicated in several neurological disorders, such as schizophrenia and Phelan-McDermid Syndrome (PMS). The association of SULT4A1 with schizophrenia was first reported in transmission disequilibrium studies. A microsatellite marker (D22s1749E) in the 5'-UTR of SULT4A1 was linked to schizophrenia development and later studies showed several intronic polymorphisms were associated with a worse psychopathology and poorer performance in cognition tests (Brennan and Condra, 2005; Meltzer et al., 2008). PMS is caused by deletions to the distal long arm of chromosome 22 (22q13.3 deletion syndrome) and is characterized by global developmental delay, intellectual disabilities, and autistic-like behaviors (Phelan, 2008). Approximately 30% of patients with PMS have a deletion encompassing SULT4A1 (Sarasua et

al., 2014). Patients <4 years of age with a SULT4A1 deletion were shown to have lower developmental quotients than with patients having two intact SULT4A1 alleles (Zwanenburg et al., 2016). Recently, nine patients were documented with interstitial 22q13.3 deletions, SULT4A1 was among the genes within the minimal critical region of all patients examined in this study. Interestingly, these patients presented with similar symptoms to PMS, including developmental and speech delays (Disciglio et al., 2014). Currently, it is unclear the extent to which SULT4A1 contributes to the pathology observed in these nine patients, although SULT4A1 was singled out as a potential candidate gene for this disorder (Disciglio et al., 2014)

Due to the high homology of SULT4A1 between species and the apparent absence of *in vitro* enzymatic activity, animal models represent an excellent system to investigate the functional role of SULT4A1 *in vivo*. Our lab recently reported that transient knockdown (KD) of zebrafish SULT4A1 using morpholino (MO) oligomers in 72-hour post fertilization (hpf) embryos resulted in an up-regulation of phototransduction genes (Crittenden et al., 2014). These genes were related to cone function and were the first identified cellular process associated with SULT4A1. Subsequent studies using TALEN-induced SULT4A1 knock-out (KO) zebrafish models, homozygous SULT4A1-KO adult zebrafish were shown to have an activity suppression phenotype during daylight hours as compared to wild-type (WT) zebrafish (Crittenden et al., 2015). The homozygous SULT4A1-KO zebrafish appeared normal, were able to readily reproduce and possessed normal lifespans (Crittenden et al., 2015). Like humans, zebrafish are diurnal organisms and are more active during daylight hours. While these observations represent the first biological pathways associated with SULT4A1, zebrafish brains lack several key brain regions characteristic of mammals, such as a cerebral cortex and an expanded telencephalon (Parker et al., 2013). Currently, there is no mammalian *in vivo* model developed to investigate the function of SULT4A1. Therefore, CRISPR-Cas9 was used to generate two distinct SULT4A1 mutant mouse models. Homozygous SULT4A1-deficient mice exhibited a progressive and severe

neurological deterioration resulting in death prior to adulthood and immunoblotting demonstrated a lack of detectable SULT4A1 expression in these mice. Interestingly, subcellular fractionation of WT brain homogenates showed SULT4A1 localized to mitochondrial, cytosolic, and microsomal fractions, but not to nuclei. Finally, SULT4A1 protein expression and localization was validated in primary cortical neuron cultures, an important *in vitro* model system for future molecular and biochemical studies. The behavioral phenotype and lethality in post-natal mice that lack functional SULT4A1 suggests that this orphan enzyme is necessary for normal neurological development during post-natal development.

## Materials and Methods

**Generation of SULT4A1 mouse models.** SULT4A1 mutant mice were custom generated by the University of Alabama at Birmingham (UAB) Transgenic Facility using CRISPR-Cas9 gene editing technology in a C57BL/6J mouse background (details in **Supplemental Methods**). Exon 1 ( $\Delta 28$ ) was targeted to generate a SULT4A1 mutation, while Exon 3 ( $\Delta 12$ ) was targeted to disrupt the structure around the active site His<sup>111</sup>. Female founders were used to generate breeding colonies for each model to generate SULT4A1 WT, heterozygous, and homozygous mutants for this study. Genotyping primers were as follows:  $\Delta 28$  Forward (5'-TCGGCCTGTAAAC-3') and Reverse (5'-GAGAGCAAGTACT-3') and  $\Delta 12$  Forward (5'-CAGATGGCTTACG-3') and Reverse (5'-TGCTCAACTGTGA-3'). Breeding colonies were maintained in air-filtered cages under barrier conditions and kept on a 12 hr light/dark cycle with *ad libitum* access to food and water. Breeding colonies were maintained in accordance with the UAB Institutional Animal Care and Use Committee (IACUC) guidelines and all procedures were approved by the UAB Committee on Animal Research.

**Subcellular fractionation of brain tissue.** Brain tissue from male mice were immediately harvested after euthanasia from day 25 old  $\Delta 28$  and  $\Delta 12$  SULT4A1 WT, heterozygous, and

homozygous male mice and washed in ice-cold PBS. Tissue was snap-frozen in liquid N<sub>2</sub> and stored at -80°C. Frozen brain tissue was placed in chilled subcellular fractionation buffer (20 mM HEPES pH 7.4, 10mM KCL, 2 mM MgCl<sub>2</sub>, 1mM of EDTA, and 1 mM EGTA) with 1 mM DTT and Complete Mini protease inhibitor (Roche Diagnostics, Indianapolis, IN) added immediately prior to use. A Potter-Elvehjem homogenizer was used to generate crude cell homogenates. Subcellular fractionation procedure was performed as follows: Brain homogenates were subjected to centrifugation at 720 x g to isolate the nuclear pellet. The supernate was centrifuged at 6,200 x g to separate the mitochondrial pellet. The nuclear and mitochondrial pellets were washed twice in subcellular fractionation buffer. The cytosolic fraction was isolated after centrifugation at 100,000 x g for 1 hr, while the resulting microsomal fraction was washed and re-centrifuged. Protein concentrations were determined by Bradford assay and standard curves were generated using 1 mg/ml gamma globulin. Subcellular fractions (50 µg) were separated by SDS-PAGE (12% acrylamide) gel electrophoresis and transferred to nitrocellulose. SULT4A1 protein was detected using rabbit anti-SULT4A1 polyclonal IgG (1:1000; Proteintech, Chicago, IL) and β-actin (1:1000; Cell Signaling, Danvers, MA) was used as a loading control, followed by a goat anti-rabbit horseradish peroxidase-conjugated secondary Ab (1:55,000, Southern Biotech, Birmingham, AL), and developed with SuperSignal West Chemiluminescent Substrate (Thermo Scientific, Waltham, MA). Purity of fractions were determined by reprobng with the following antibodies: 1). Nuclear (Histone H3 1:2500 rabbit polyclonal, Cell Signaling, Danvers, MA) 2). Mitochondria (Tom20 1:1000 rabbit polyclonal, Proteintech, Chicago, IL) 3) Cytosol (GAPDH 1:1000 rabbit monoclonal, Cell Signaling, Danvers, MA) 4) Microsomes (Na<sup>+</sup>/K<sup>+</sup> ATPase 1:1000 rabbit polyclonal, Cell Signaling, Danvers, MA).

**Generation of primary neuronal cultures.** Primary cortical neuronal cultures were prepared from gestational day 15 CD-1 mice. Briefly, the cortical regions of the embryonic brains were aseptically dissociated, freed of meninges and dissociated in MEM medium containing 10% FBS.

Cortical neurons were cultured in Neurobasal medium (Thermo Fisher Scientific, Waltham, MA) supplemented with B27, sodium pyruvate and 2% horse serum. Cells were plated to a density of  $5 \times 10^5$  in 6-well plate (Nalge Nunc International, Rochester, NY) previously coated with 0.1 mg/ml sterile poly-L-ornithine (Sigma-Aldrich, St. Louis, MO). The cultures were maintained at 37°C in 5% CO<sub>2</sub> and in a humidified incubator. On day *in vitro* (DIV) 3, the cultures were treated with 5-fluoro-2-deoxyuridine (40 μM) to inhibit glial growth and proliferation. Experiments were performed at DIV 12. Under these conditions, mature neurons represent greater than 95% of the cells in the culture.

**Immunocytochemistry.** For immunofluorescence studies, primary cortical neurons were grown on poly-L-ornithine coated coverslips for 12 days. Cells were washed twice with ice-cold phosphate buffered saline (PBS) then fixed in 4 % (w/v) paraformaldehyde for 20 min at room temperature. Next, cells were washed three times with PBS and permeabilized by using 0.2% (v/v) Triton X-100 in 10% donkey serum for 1 hr. The permeabilized cells were then incubated with primary Ab (SULT4A1 1:500, Proteintech, Chicago, IL) overnight at 4°C. After incubation with primary Ab, cells were washed with 1x PBS and incubated with the secondary antibody conjugated with Alexa Fluor 555 (Invitrogen) [1:1500 in 1% (w/v) Donkey serum in PBS] in dark for 1 hr at room temperature. Nuclei were visualized using Hoechst (ThermoScientific, Waltham, MA) diluted in PBS (1:20,000). Finally, cells were washed with PBS and mounted onto a glass slide using Immuno-Mount (ThermoScientific). Cells were visualized and images were captured using Nikon confocal microscope with a 100 1.35 NA objective (Wetzler, Hassen, Germany).

## Results and Discussion

SULT4A1 remains an orphan SULT with an unknown function. In zebrafish eyes, SULT4A1 mRNA was detected by RT-qPCR in 72 hpf embryos and MO-mediated SULT4A1 KD resulted in up-regulation of phototransduction genes related to cone function (Crittenden et al.,



2014). TALEN-induced SULT4A1 KO adult zebrafish were shown to be less active during daylight hours, yet appeared normal, reproduced, and thrived (Crittenden et al. 2015). To determine the function of SULT4A1 in a mammalian system, CRISPR-Cas9 was used to mutate/delete SULT4A1 in C57BL/6J mice. Analysis of founder mice yielded two female mice with SULT4A1 mutations of interest and were selected for breeding. The first founder possessed a 28 bp deletion ( $\Delta 28$ ) in exon 1 of the SULT4A1 gene causing a frameshift mutation beginning at amino acid (AA) 46 and premature stop codon 16 AA downstream. (**Figure 1A**). The second founder female carried a 12 bp ( $\Delta 12$ ) in-frame deletion on exon 3 of the SULT4A1 gene (**Figure 1B**). These four AAs (L-I-K-S) form a  $\beta$ -sheet immediately adjacent to the active site His<sup>111</sup> and structurally align the His within the substrate binding pocket. Although SULT4A1 lacks sulfonation activity *in vitro*, a possibility remains that the active site His<sup>111</sup> is integral to its function *in vivo*. Furthermore, dynamic modeling suggested the 4 AA in-frame deletion would displace the active site His<sup>111</sup> in such a manner as to be non-functional.

Heterozygous mice of both strains appeared to develop normally to adulthood, reproduced without noticeable deficits, and lacked any readily observable phenotype. Interestingly, select offspring from heterozygote breeding pairs of both the  $\Delta 28$  and  $\Delta 12$  mice appeared normal at birth; however 8-10 days post-natal, several of the pups developed a slight tremor. As these pups aged, tremor increased in intensity and other symptoms appeared including absence seizures, abnormal gait, ataxia, decreased weight gain compared to WT and heterozygous littermates. Symptoms progressed in intensity until the mice were unable to move or feed and were euthanized according to IACUC guidelines around post-natal days 21-25. Videos of day 17  $\Delta 28$  (**Supplemental Video 1**) and  $\Delta 12$  (**Supplemental Video 2**) homozygous pups exhibiting some of the neurological phenotypes are included in the supplementary data. DNA sequencing confirmed the pups exhibiting the phenotype were  $\Delta 28$  and  $\Delta 12$  homozygous mutants and possessed the same genomic deletions as the founder females (**Supplemental Figure 1**). The

neurological phenotype is consistent in all homozygous pups for both strains. Since homozygotes do not reach adulthood, these pups must be generated by mating heterozygous SULT4A1 mutant male and females.

Homozygous SULT4A1 mutant mice presenting with severe neurological deficits was an unexpected novel finding. Based on the behavioral phenotype of SULT4A1-KO zebrafish and the lack of effect on breeding, morphology, or lifespan, the SULT4A1 mutant mice were not predicted to exhibit a severe neurological phenotype. Extensive behavioral testing was anticipated to identify a behavioral phenotype in mice. The lethality of the pups during post-natal development suggests SULT4A1 has a critical role in mammalian post-natal brain development. Primary symptoms manifested by both  $\Delta 28$  and  $\Delta 12$  homozygous mice were tremor, rigidity, and seizure. In general, seizure disorders are thought to manifest due to an imbalance between neuronal excitation and inhibition, that leads to hyperexcitability and manifestation of a seizure (Scharfman, 2007). While not the singular cause, functional SULT4A1 would seem to be a critical protein to the homeostatic regulation of balanced neuronal signaling. Further studies are needed to fully characterize this behavioral phenotype in mice with absent or non-functional SULT4A1. Due to the severe phenotype, heterozygote mice of both strains will be carefully analyzed to determine whether loss of one functional SULT4A1 allele results in haploinsufficiency.

To confirm if homozygous mutant pups lacked SULT4A1, cytosol was prepared from male brains of each genotype and immunoblotted to determine SULT4A1 protein levels in both the strains. As shown in **Figure 2A**, for both strains, heterozygotes showed a modest decrease in SULT4A1 protein levels as compared to WT littermates. For  $\Delta 28$  and  $\Delta 12$  homozygous mutant pups, SULT4A1 protein was not detectable by immunoblotting. The lack of SULT4A1 staining in the  $\Delta 12$  homozygous samples was surprising based on dynamic modeling studies (data not shown). Multiple antibodies were used to probe  $\Delta 12$  homozygous mutant brain samples and the

results showed a consistent lack of immunostaining suggesting rapid SULT4A1 protein degradation.

To date, no sulfonation activity has been reported for SULT4A1 in the literature. In addition, a thorough investigation of the cellular location of SULT4A1 in brain has not been reported. Therefore, WT male mouse brain homogenates were subjected to subcellular fractionation via differential centrifugation to investigate the localization of SULT4A1. In addition to a cytosolic localization, SULT4A1 was detected in both mitochondrial and microsomal fractions (**Figure 2B**). Nuclear, mitochondrial, and microsomal pellets were washed twice and resuspended to ensure purity of the fraction. The mechanism of SULT4A1 localization to these fractions remains unknown; however, several possibilities exist to explain this occurrence. First, it is possible that SULT4A1 has a binding partner that localizes to the mitochondrial or microsomal fraction. A second possibility involves an unknown post-translational modification, altering the localization of SULT4A1. This is not unprecedented as the Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1 protein (PIN1) was shown to bind to SULT4A1 by co-immunoprecipitation (Co-IP) studies and binding negatively regulated SULT4A1 protein stability (Mitchell and Minchin, 2009). SULT4A1 was shown to be phosphorylated by ERK1 on a Thr residue, which facilitated PIN1 binding and increased SULT4A1 degradation (Mitchell et al., 2011). Importantly, these experiments were carried out *in vitro* and the *in vivo* significance of this interaction in neurons is still unknown. Future Co-IP studies will be conducted to determine binding partners in each fraction using mouse brain and/or fetal cortical neurons.

Active SULT4A1 protein requires proper splicing, since reports indicate SULT4A1 mRNA is detectable in various non-neuronal tissues, but is not correctly spliced and is nonfunctional (Falany et al., 2000; Sidharthan et al., 2014). Low levels of SULT4A1 have been reported to be endogenously expressed by immunoblot in established cell lines, including LN229 glioblastoma and SH-SY5Y neuroblastoma cells (Sun et al., 2012; Sidharthan et al., 2014). The question

remains if these immortalized cancer cell lines recapitulate the neuronal environment in which SULT4A1 exerts its normal function, as this protein is found to be highly localized to neurons. Therefore, the expression and localization of SULT4A1 in primary mouse cortical neuron culture was investigated, as these primary cultures may provide a more suitable *in vitro* model to study SULT4A1. Primary cortical neurons were harvested on gestational day 15 CD-1 mice and grown on coverslips for 12 days to become enriched for neurons. **Figure 3** shows SULT4A1 (green) is highly expressed in DIV 12 primary cortical neurons, indicating SULT4A1 is present early in embryonic neuron development. In addition, SULT4A1 immunoreactivity is absent in the nuclei (blue), but is detected in neurite projections, as well as, the cell body of the neuron. Although the SULT4A1 mutant mice were generated in a C57BL/6J and these primary cultures were derived from CD-1 mice, it is expected to be readily reproducible in our strain. SULT4A1 expression was also detected in mitochondrial, microsomal and cytosolic fractions but not nuclei, generated from the primary cultured neurons by immunoblotting similar to pattern observed with mouse brains (data not shown).

The expression of SULT4A1 in primary neuron culture was first reported by Butcher et al. in a study investigating the transcriptional regulation of SULT4A1. The authors showed that both SULT4A1 mRNA and protein were detected in primary neuron cultures derived from embryonic day 16 mice, although the subcellular localization was not reported (Butcher et al., 2010). Interestingly, SULT4A1 was expressed in the neurite projections of primary cortical neurons (**Figure 3**). The impact of SULT4A1 on neurite growth and guidance remains unknown. Evidence of SULT4A1 expression in embryonic neurons is a promising model system to elucidating the mechanistic function of SULT4A1 *in vitro*. It is reasonable to infer that primary neuron cultures better represent the appropriate molecular environment than immortalized tumor-derived cell lines. Breeding pairs are established for the production of  $\Delta 28$  and  $\Delta 12$  WT, heterozygous and homozygous primary neuron cultures. These studies in conjunction with behavioral analyses will

provide valuable insights to evaluate potential differences in neuronal maturation, synaptic formation, or synaptic transmission.

In summary, this study reports the first published generation and characterization of SULT4A1 mutant mouse models. Homozygous mutant  $\Delta 28$  and  $\Delta 12$  SULT4A1 mice present with a severe and progressive neurological phenotype resulting in post-natal death. The lack of detectable SULT4A1 in the homozygous mutant pups by immunostaining suggests that this is an essential protein required for normal development. In addition, although SULT4A1 was thought to be a cytosolic protein, its expression was detected in mitochondrial and microsomal fractions indicating potential novel function(s). SULT4A1 was also found to be expressed in embryonic mouse brain further suggesting an essential role in development. Although, the function of SULT4A1 remains unknown, these mouse models will be invaluable to identifying characterizing the role of SULT4A1 in mammalian brain.

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### **Authorship Contributions**

**Participated in research design: Garcia, Hossain, Andrabi, Falany**

**Conducted experiments: Garcia, Hossain**

**Performed data analysis: Garcia, Hossain, Andrabi, and Falany**

**Wrote or contributed to the writing of the manuscript: Garcia, Hossain, Andrabi, and Falany**

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## Footnotes

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## Figure legends

**Figure 1.** Schematic representation of SULT4A1 mutations generated by CRISPR-Cas9. A.) Diagram of the  $\Delta 28$  mutant SULT4A1 mouse line. CRISPR-Cas9 technology induced a 28 bp deletion within exon 1 that resulted in a frameshift mutation and premature stop codon at AA 62. B.) Diagram of the  $\Delta 12$  mutant SULT4A1 mouse line. Gene editing induced a 12 bp in-frame deletion of the 4 AA immediately preceding the active site His. For both lines, SULT4A1 female founders were used to establish breeding colonies.

**Figure 2.** Characterization of SULT4A1 protein expression in  $\Delta 28$  and  $\Delta 12$  mutant mouse brains and subcellular localization of WT SULT4A1. WT, heterozygous, and homozygous  $\Delta 28$  and  $\Delta 12$  D25 old mice were euthanized and whole brains were harvested. A.) WT, heterozygous (HET), and homozygous (MUT)  $\Delta 28$  and  $\Delta 12$  mice were probed for SULT4A1 protein expression. Human brain cytosol was used as a positive control (CTRL) for SULT4A1. For both strains, WT and heterozygotes showed positive staining for SULT4A1; however,  $\Delta 28$  and  $\Delta 12$  homozygotes were negative for SULT4A1. B.) Subcellular fractionation was used to generate nuclear (Nuc), mitochondrial (Mito), cytosolic (Cyto) and microsomal (Micro) fractions from WT adult mouse brain homogenates. Aliquots of each fraction (50  $\mu$ g) were subjected to SDS-PAGE and immunoblotted for SULT4A1. To determine the purity of subcellular fractions, marker proteins specific to each fraction were probed. Histone H3 was used to probe nuclear fraction purity, TOM20 was used as a marker protein for the mitochondrial fraction, GAPDH was used as a marker for the cytosolic fraction, and Na<sup>+</sup>/K<sup>+</sup> ATPase was used to determine the microsomal fraction purity. SULT4A1 protein was detected in the mitochondrial, cytosolic, and microsomal fractions and marker proteins demonstrated pure subcellular fractions.

**Figure 3.** SULT4A1 expression in embryonic-derived mouse cortical neurons by confocal microscopy. Cortical neurons were harvested from embryonic day 15 mice and grown on

coverslips for an additional 12 days prior to immunocytochemistry. Cortical neurons were probed for SULT4A1 expression using a rabbit polyclonal igG at a 1:5000 dilution. Nuclei were labeled with Hoechst (1:20,000) with Primary cortical neurons are positive (green) for SULT4A1 protein in both the soma and neurites, but not in the nuclei (blue).

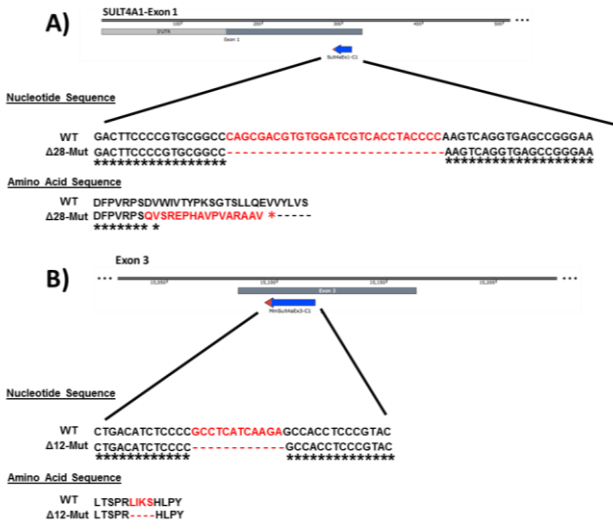


Figure 1

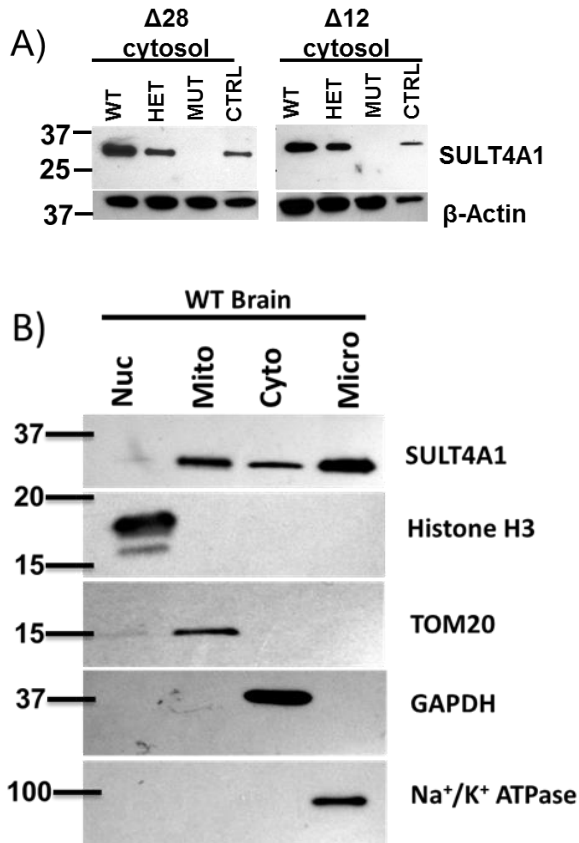


Figure 2

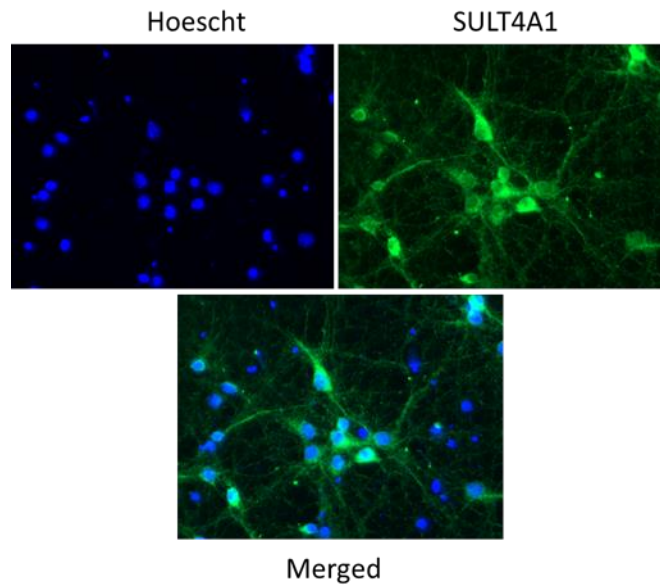


Figure 3